Angiotensin type 1 and type 2 receptor blockade in chronic allograft nephropathy

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Angiotensin-II (Ang-II) type 1 (AT_1) receptor blockers may delay the progression of chronic allograft nephropathy (CAN). However, neither the optimal time for initiating AT_1 receptor blockade in order to delay CAN potentially nor the role of Ang-II type 2 (AT_2) receptors under AT_1 receptor blockade is known. Both AT receptors can regulate p53 expression and apoptosis. We investigated what time of initiation with AT_1 blockers most effectively delayed CAN as well as the role of the AT_2 receptor, and how angiotensin receptor blockade affected apoptosis and its regulating factors in this context in a rat model. Kidneys of Fisher (F344) rats were transplanted into Lewis rats. Animals were treated with AT_1 (candesartan) and/or AT_2 (PD123319) receptor antagonists, a calcium channel blocker, or vehicle (treatment periods: day −7 before to week 24 after transplantation (long term), week 12 to week 24 (late), day −7 to day +5 (early)) and observed the animals for 24 weeks. Reduction of proteinuria, grade of CAN, and number of apoptotic cells was most pronounced in animals receiving long-term AT_1 receptor blockade. A combined AT_1/AT_2 blocker treatment reduced CAN similarly to AT_1 blocker treatment alone. The number of apoptotic cells and the level of p53 mRNA were significantly lower in long-term AT_1 blocker-treated animals. In summary, AT_1 receptor blockade delayed the progression of CAN, particularly in animals treated long term. Reduction of apoptosis could be related to these beneficial effects. The AT_2 receptor does not appear to play an important role in CAN.

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Chronic allograft nephropathy (CAN), a major cause of late allograft failure, is characterized by glomerulopathy, tubular atrophy, interstitial fibrosis, and vasculopathy.[1,2] The initial graft damage is mediated by ischemia/reperfusion, surgical trauma, and alloantigen-related factors, thus influencing the development and progression of CAN.[3,4]

Inhibition of the renin–angiotensin system by angiotensin-converting enzyme inhibitors[5] and Angiotensin-II (Ang-II) type 1 (AT_1) receptor blockers[6] ameliorates the progression of chronic renal diseases including CAN.[7] However, the optimal time point for the initiation of AT_1 receptor blockade to achieve the best amelioration of long-term graft function is as unknown as the contribution of the AT_1 and the Ang-II type 2 (AT_2) receptors in this context.

Among other effects, signalling via the AT_1 receptor increases blood pressure, and promotes cellular proliferation as well as extracellular matrix production, thus contributing to the progression of fibrosis. The role of the AT_2 receptor on the other hand, which could be involved in antiproliferative, antiangiogenic, and blood pressure lowering effects during the development of CAN, is not yet clear. Thus, the amelioration of fibrosis in CAN by AT_1 receptor blockade may also depend on signalling via non-blocked AT_2 receptors.

Furthermore, the mechanisms involved in the protective effects of AT_1 receptor blockade are not fully understood.

Apart from reduced proteinuria and proliferation, a reduced number of apoptotic cells in the graft may be related to the amelioration of CAN by AT_1 receptor antagonists. Apoptosis is an intrinsically controlled process of cellular suicide[8] and can significantly contribute to tissue damage by a reduction of functioning kidney cells.[9] An increased number of apoptotic cells has been described in human graft biopsies with CAN,[10] whereas a reduced expression of cytoprotective factors together with an increased number of apoptotic cells has been observed in a rat model of CAN.[11] Interestingly, apoptosis can be induced by Ang-II via both AT_1[12,13] and AT_2[14–16] receptors in various cell types, including renal tubular cells.[17,18] These divergent results could be related to different cell types and treatment conditions.

How Ang-II influences apoptosis is not fully understood. A potential factor involved in Ang-II-mediated apoptosis is

p53, which can result in cell cycle arrest and/or apoptosis.[19] Stimulation of AT_1 receptors increased p53 in parallel to the number of apoptotic cells in a model of stretch-induced stress in myocytes.[20] AT_1 and AT_2 receptors have been reported to stimulate p53 DNA binding or p53 expression, respectively, together with an upregulation of the apoptosis-promoting Bax protein.[21,22]

Here, we analyzed the effects of AT_1 and AT_2 receptor blockade over different periods after kidney transplantation on the progression of CAN, the role of the AT_2 receptor during CAN, and how AT receptor blockade influences apoptosis and the expression of p53 as a possible link between Ang-II and apoptosis regulation.

RESULTS

Long-term treatment with an AT_1 receptor antagonist resulted in reduced proteinuria and morphological changes of CAN

Proteinuria and creatinine clearances 24 weeks after transplantation. Animals receiving *long-term* AT_1- (AT1) and combined AT_1/AT_2 antagonist treatment (AT1 + AT2) developed a significantly lower proteinuria than animals receiving AT_2 antagonist- (AT2) and vehicle (VEH) treatment ($P \leqslant 0.03$; Figure 1a), whereas proteinuria tended to be lower as compared to animals treated with a calcium channel blocker (CCB) ($P = 0.06$ and 0.11). Animals receiving *late* and *early* treatment developed no statistically significant differences ($P > 0.05$; Figure 1b and c), although animals receiving late AT_1- (AT1) and combined AT_1-/AT_2 antagonist treatment (AT1 + AT2) had lower proteinuria as animals of groups AT2, CCB, and VEH.

Creatinine clearances as well as mean arterial pressure and body weights did not differ significantly between the groups at week 24 (Table 1).

One animal of each group AT1, AT2, and VEH receiving *long-term* treatment died owing to surgical complications; one animal of group VEH died after week 12.

One animal of group AT1 receiving *late* treatment died after surgery. One animal from each group AT1, AT2, and AT1 + AT2 died after 20 weeks.

One animal of group AT2 receiving *early* treatment died after 12 and 20 weeks, respectively.

Grade of CAN. Animals of *long-term* treatment group AT1 or group AT1 + AT2 developed a significantly lower grade of CAN as compared to the groups AT2, CCB, and VEH ($P < 0.05$; Figure 2a). Animals treated *late* or *early* did not significantly differ in the grade of CAN. However, animals receiving late treatment of groups AT1 as well as group AT1 + AT2 had, although not significant, lower grades of CAN ($P > 0.05$; Figure 2b and c).

Glomerulosclerosis. Glomerulosclerosis was significantly lower in animals treated *long term* with the AT_1 receptor antagonist as compared to groups AT2, CCB, and VEH ($P \leqslant 0.012$; Table 1, Figure 3a). Animals of group AT1 + AT2 had a lower glomerulosclerosis as compared to the groups AT2, CCB, and VEH ($P \leqslant 0.025$). Glomerulosclerosis in animals receiving *late* and *early* treatment did not significantly differ, although, again, late treatment in groups AT1 and AT1 + AT2 resulted in a lower glomerulosclerosis as compared to the groups AT2, CCB, and VEH ($P > 0.05$; Table 1).

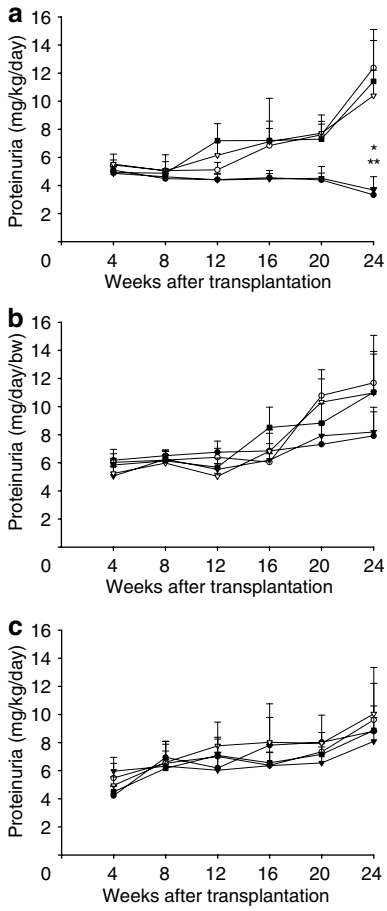

Figure 1 | Proteinuria 24 weeks after transplantation. (**a**) Proteinuria in animals treated *long term* from day −7 before transplantation to week 24 after transplantation was significantly lower in animals of groups AT1 and AT1 + AT2 as compared to groups AT2 and VEH (*AT1 vs AT2: $P = 0.03$; vs CCB: $P = 0.06$; vs VEH: $P = 0.03$. **AT1 + AT2 vs AT2: $P = 0.006$; vs CCB: $P = 0.11$; vs VEH: $P = 0.024$). (**b**) Proteinuria in animals treated *late* from week 12 to week 24 after transplantation was lowest in animals of groups AT1 and AT1 + AT2. However, the differences were not statistically significant (*$P > 0.05$). (**c**) Animals treated *early* from day −7 before transplantation to day 5 after transplantation had no significantly different proteinuria ($P > 0.05$). ● = AT1: treatment with AT_1 receptor antagonist; ○ = AT2: treatment with AT_2 receptor antagonist; ▼ = AT1 + AT2: treatment with AT_1 and AT_2 receptor antagonist; ▽ = CCB: treatment with CCB; and ■ = VEH: treatment with vehicle.

Macrophage infiltration of the graft. Animals of *long-term* treatment group AT1 had a significantly reduced number of macrophages in graft tissues as compared to group AT2 ($P = 0.01$). Macrophage infiltration was also lower as compared to groups CCB and VEH ($P < 0.03$; Table 1). Animals of group AT1 + AT2 had a significantly reduced macrophage infiltration as compared to groups AT2, CCB, and VEH ($P < 0.01$). *Late* and *early* treatment resulted in no

Table 1 | Body weight (g; mean ± s.e.m.), MAP (mm Hg; mean ± s.e.m.), and creatinine clearance (ml/min; mean ± s.e.m.)

Group	AT1	AT2	AT1+AT2	CCB	VEH
Long-term treatment (n)	7	7	8	8	6
Body weight (g)	480.6 ± 19.9	474.0 ± 12.6	488.8 ± 9.6	488.0 ± 9.7	497.5 ± 9.8
MAP (mm Hg)	92.6 ± 3.9	95.2 ± 6.6	86.7 ± 4.1	89.8 ± 6.4	92.5 ± 6.6
Creatinine clearance (ml/min/kg)	4.01 ± 0.37	4.09 ± 0.36	4.02 ± 0.43	4.66 ± 0.55	3.78 ± 0.24
Glomerulosclerosis (%)	11.5 ± 1.3°	24.5 ± 1.8	16.5 ± 1.4°°	27.5 ± 2.0	27.7 ± 1.6
Grade of macrophage infiltration	◆		◆◆		
0	5	—	4	—	—
Grade 1	2	1	4	4	1
Grade 2	—	4	—	2	2
Grade 3	—	2	—	2	3
PCNA (cells/field of view)	4.9 ± 0.6	5.5 ± 0.8	5.7 ± 1.1	4.1 ± 0.5	4.5 ± 0.5
Late treatment (n)	6	7	7	8	8
Body weight (g)	467.0 ± 20.2	472.5 ± 9.4	474.3 ± 19.4	479.3 ± 6.3	479.1 ± 11.9
MAP (mm Hg)	93.7 ± 8.1	91.3 ± 7.1	88.8 ± 5.8	94.8 ± 8.5	95.4 ± 5.9
Creatinine clearance (ml/min/kg)	4.02 ± 0.34	4.17 ± 0.41	3.67 ± 0.36	3.67 ± 0.95	4.18 ± 0.22
Glomerulosclerosis (%)	18.5 ± 2.4	23.4 ± 3.1	18.1 ± 1.7	21.1 ± 2.1	22.1 ± 1.5
Grade of macrophage infiltration					
0	3	—	3	1	1
Grade 1	2	2	3	3	3
Grade 2	1	3	1	3	2
Grade 3	—	2	—	1	2
PCNA (cells/field of view)	4.2 ± 2.2	4.1 ± 1.8	4.9 ± 0.8	4.4 ± 0.7	4.0 ± 2.4
Early treatment (n)	6	4	6	6	6
Body weight (g)	472.0 ± 13.5	482.5 ± 18.3	484.7 ± 8.5	486.7 ± 9.0	460.5 ± 16.6
MAP (mm Hg)	94.7 ± 4.2	94.0 ± 10.1	91.6 ± 4.8	91.0 ± 8.8	93.6 ± 6.9
Creatinine clearance (ml/min/kg)	4.23 ± 0.3	4.12 ± 0.33	3.78 ± 0.22	3.85 ± 0.38	4.01 ± 0.24
Glomerulosclerosis (%)	23.6 ± 3.1	25.8 ± 3.3	27.4 ± 3.2	25.6 ± 3.9	22.9 ± 2.1
Grade of macrophage infiltration					
0	1	—	2	1	1
Grade 1	2	1	2	3	2
Grade 2	1	2	2	2	2
Grade 3	2	1	—	—	1
PCNA (cells/field of view)	4.9 ± 1.0	5.9 ± 1.4	5.0 ± 0.9	5.7 ± 0.7	5.0 ± 0.4

AT_1, Angiotensin-II type 1; AT_2, Angiotensin-II type 2; CCB, calcium channel blocker; MAP, mean arterial pressure; PCNA, proliferating cell nuclear antigen; VEH, vehicle.
Glomerulosclerosis of graft tissue of the experimental groups: *long-term* treatment: °P=0.006 vs AT2, P=0.006 vs CCB, and P=0.012 vs VEH; °°P=0.012 vs AT2, P=0.012 vs CCB, and P=0.024 vs VEH. *Late* and *early* treatment: $P > 0.05$ AT1 and AT1+AT2 vs AT2, CCB, and VEH.
Grade of infiltration by macrophages (CD68 positive cells): 0=<5 cells/field of view; grade 1=6–25 cells/field of view; grade 2=26–50 cells/field of view; and grade 3=>50 cells/field of view. Numbers represent number of cases with the respective grade of infiltration: *Long-term* treatment: ◆P=0.01 vs AT2, P=0.028 vs CCB, and P=0.016 vs VEH; ◆◆P=0.008 vs AT2, P=0.006 vs CCB, and P=0.013 vs VEH. *Late* and *early* treatment: $P > 0.05$ AT1 and AT1+AT2 vs AT2, CCB, and VEH.
Number of proliferating cells (PCNA) (cells/field of view; mean ± s.e.m.).
Long-term treatment (day −7 to week 24), late treatment (week 12 to week 24), and early treatment (day −7 to day +5); *n*=number of animals analyzed 24 weeks after transplantation.

significant differences. However, animals of late treatment group AT1 and AT1 + AT2 had a reduced number of graft-infiltrating macrophages as compared to groups AT2, CCB, and VEH, ($P > 0.05$; Table 1).

Number of proliferating cells. The number of proliferating cells did not differ significantly between the experimental groups at the end of the observation period 24 weeks after transplantation (Table 1).

Long-term treatment with an AT_1 receptor antagonist reduced the number of apoptotic cells

Number of apoptotic cells. Animals of *long-term* treatment groups AT1 and AT1 + AT2 had a significantly lower number of apoptotic cells in their grafts as compared to animals of group AT2 and VEH ($P \leqslant 0.03$; Figures 3b and 4a).

The number of apoptotic cells in grafts of animals of *late and early* treatment groups AT1 or AT1 + AT2 were not significantly different as compared to groups AT2, CCB, and VEH ($P > 0.05$; Figure 4b and c).

Levels of mRNA of apoptosis-related factors bcl-2/bax mRNA ratio. The lower number of apoptotic cells in animals of *long-term* treatment group AT1 was paralleled by a significant shift of the mRNA ratio of bcl-2/bax towards bcl-2 mRNA (coding for apoptosis-inhibiting Bcl-2) in group AT1 as compared to groups AT2 ($P = 0.045$) and VEH ($P = 0.012$), as well as group AT1 + AT2 as compared to group VEH ($P = 0.018$; Figure 5a).

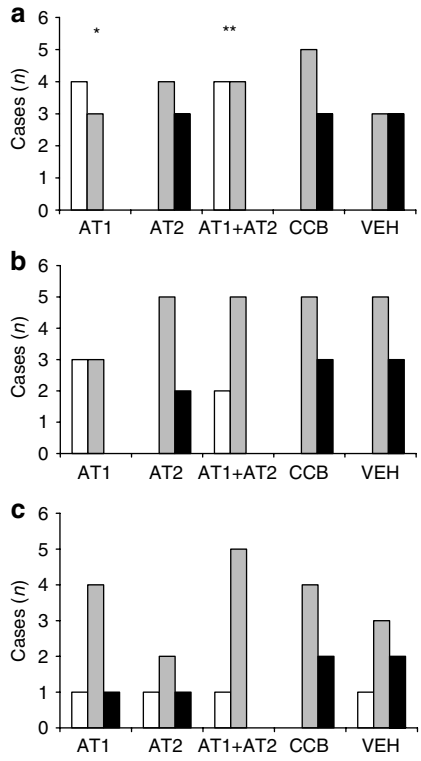

Figure 2 | Chronic allograft nephropathy. (**a**) Grade of CAN in animals treated *long term* from day −7 before transplantation to week 24 after transplantation was significantly lower in animals of groups AT1 and AT1 + AT2 as compared to the other groups (*AT1 vs AT2: $P = 0.028$; vs CCB: $P = 0.024$; vs VEH: $P = 0.031$. **AT1 + AT2 vs AT2: $P = 0.031$; vs CCB: $P = 0.029$; vs VEH: $P = 0.03$). (**b**) Grade of CAN in animals treated *late* from week 12 to week 24 after transplantation tended to be lower in animals of groups AT1 and AT1 + AT2 as compared to the other groups ($P > 0.05$). (**c**) Grade of CAN in animals treated *early* from day −7 before transplantation to day 5 after transplantation did not significantly differ between the groups ($P > 0.05$). Grade of CAN: □ = mild, ▒ = moderate, and ■ = severe.

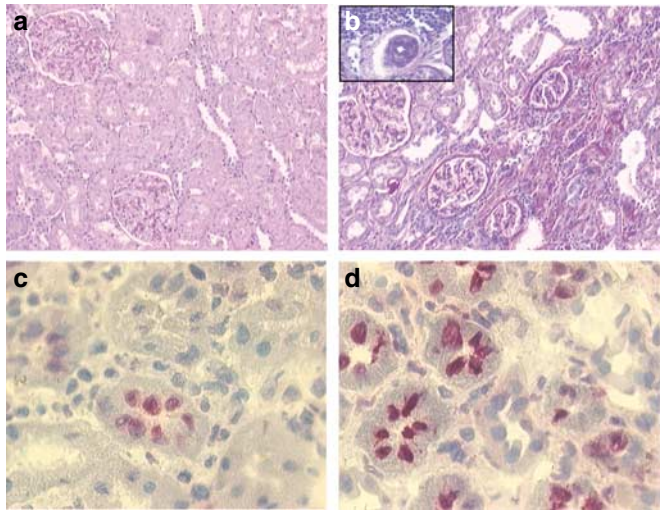

Figure 3 | Histology of kidney grafts. (**a**) Normal tubuli and glomeruli in animals of group AT1 (long term); (**b**) interstitial fibrosis, tubular atrophy, glomerulosclerosis, and inflammatory infiltrates in long-term control animals (VEH) (**a** and **b** periodic acid-Schiff (PAS) reaction, original maginification × 100); small artery with hypertrophy and hyperplasia of the intima and media (PAS reaction, original magnification × 400) (see inset). (**c**) Apoptotic tubular cells in graft tissue of animals receiving long-term AT_1 antagonist treatment (AT1) (original magnification × 400); (**d**) TUNEL-positive tubular cells in graft tissue of long-term controls (VEH) (original magnification × 400). Apoptosis primarily affected tubular epithelial cells of the proximal and distal tubules within the cortex as well as within nephron segments of the medulla.

A significant shift of the mRNA ratio of bcl-2/bax towards bcl-2 mRNA was observed in animals of *late* treatment groups AT1 and AT1 + AT2 ($P < 0.01$; Figure 5b), whereas *early* treatment resulted in no significant differences as compared to controls ($P > 0.05$; Figure 5c).

p53 levels. Animals of *long-term* treatment group AT1 had significantly lower p53 levels as compared to animals of groups AT2 ($P = 0.03$) and VEH ($P = 0.06$; Figure 6a). *Late* and *early* treatment resulted in no significant differences in the expression of p53 ($P > 0.05$; Figure 6b and c).

Caspase-1 and caspase-3 mRNA levels. Animals of *long-term* treatment group AT1 had significantly lower levels of caspase-1 and caspase-3 mRNA as compared to animals of groups AT2 and VEH ($P \leqslant 0.03$; Figures 7a and 8a). Animals of group AT1 + AT2 had significantly reduced caspase-3 mRNA levels as compared to animals of groups CCB and VEH ($P = 0.03$; Figure 7a), whereas caspase-1 mRNA levels were significantly lower as compared to animals of group VEH ($P = 0.012$; Figure 8a).

Animals of *late* treatment group AT1 had significantly lower caspase-1 and caspase-3 mRNA levels as compared to animals of group VEH ($P < 0.01$; Figures 7b and 8b).

Late combined AT_1 and AT_2 receptor antagonist treatment resulted in significantly lower caspase-1 mRNA levels as compared to animals of groups AT2 and VEH ($P = 0.03$; Figure 8b).

Animals receiving *early* treatment developed no significant differences (Figures 7c and 8c).

DISCUSSION

In our model of CAN, AT_1 receptor blockade reduced proteinuria, grade of CAN, and glomerulosclerosis as compared to animals receiving AT_2 receptor blockade and controls receiving VEH treatment. The beneficial effects of AT_1 receptor blockade were most obvious in animals treated for the entire period as compared to animals receiving late AT_1 antagonist treatment. Although creatinine clearances did not differ between the experimental groups, after a longer follow-up, a significant difference could be expected between the groups in this experimental model as development of proteinuria usually precedes a drop in glomerular filtration rate.

These results are in congruence with observations that inhibition of the renin–angiotensin system by angiotensin converting enzyme inhibition limits CAN even when treatment was delayed.[23] Although we also observed a

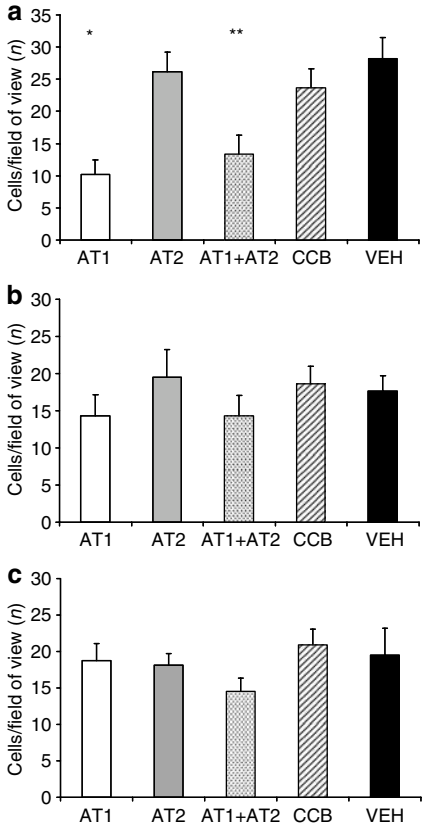

Figure 4 | TUNEL-positive apoptotic cells. (**a**) TUNEL-positive apoptotic cells in animals treated *long term* from day −7 before transplantation to week 24 after transplantation were significantly lower in animals of group AT1 and group AT1 + AT2 as compared to groups AT2 and VEH (*AT1 vs AT2: $P = 0.027$; vs CCB: $P = 0.12$; vs VEH: $P = 0.03$. **AT1 + AT2 vs AT2: $P = 0.015$; vs CCB: $P = 0.12$; vs VEH: $P = 0.03$). (**b**) Apoptotic cells in animals treated *late* from week 12 to week 24 after transplantation tended to be lower in animals of group AT1 and AT1 + AT2 as compared to the other groups ($P > 0.05$). (**c**) The number of apoptotic cells in animals treated *early* from day −7 before transplantation to day 5 after transplantation did not differ significantly between the groups ($P > 0.05$). □ = AT1: treatment with AT_1 receptor antagonist; ▩ = AT2: treatment with AT_2 receptor antagonist; ⬚ = AT1 + AT2: treatment with AT_1 and AT_2 receptor antagonist; ▨ = CCB: treatment with CCB; and ■ = VEH: treatment with vehicle.

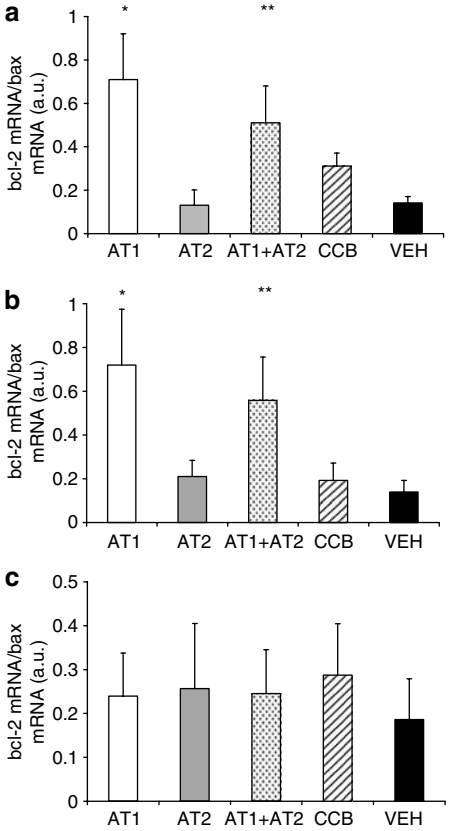

Figure 5 | Ratio of bcl-2/bax mRNA levels. (**a**) Animals receiving *long-term* AT_1 antagonist treatment (group AT1) from day −7 before transplantation to week 24 after transplantation had a significantly shifted ratio toward bcl-2 mRNA as compared to animals of groups AT2 and VEH (*AT1 vs AT2: $P = 0.045$; vs CCB: $P = 0.27$; vs VEH: $P = 0.012$). Animals of group AT1 + AT2 had a ratio, which was significantly shifted to bcl-2 mRNA as compared to group VEH (**AT1 + AT2 vs AT2: $P = 0.06$; vs CCB: $P > 0.05$; vs VEH: $P = 0.018$). (**b**) Animals receiving *late* (week 12 to week 24 after transplantation) AT_1 antagonist treatment (group AT1) and $AT_1 + AT_2$ antagonist treatment (group AT1 + AT2) had a ratio shifted towards bcl-2 mRNA as compared to animals of groups AT2, CCB, and VEH (*AT1 vs AT2: $P = 0.006$; vs CCB: $P = 0.006$; vs VEH: $P = 0.003$. **AT1 + AT2 vs AT2: $P = 0.018$; vs CCB: $P = 0.03$; vs VEH: $P = 0.009$). (**c**) The ratios in animals receiving *early* treatment from day −7 before transplantation to day 5 after transplantation did not significantly differ between the groups ($P > 0.05$). □ = AT1: treatment with AT_1 receptor antagonist; ▩ = AT2: treatment with AT_2 receptor antagonist; ⬚ = AT1 + AT2: treatment with AT_1 and AT_2 receptor antagonist; ▨ = CCB: treatment with CCB; and ■ = VEH: treatment with vehicle.

reduction in proteinuria and grade of CAN in animals with delayed treatment, this effect was less pronounced in our experiment. The differences can be explained by use of an AT_1 receptor antagonist, the shorter follow-up time, and a lower cyclosporin A dose in our series.

Whether AT_2 receptors, whose expression increases under pathological conditions,[24] contribute to the beneficial effects after the blockade of AT_1 receptors in delaying CAN is not known. Some groups suggested that signalling via the AT_2 receptor mediates the beneficial effects associated with the blockade of AT_1 receptors as AT_2 receptor knockout mice developed a more severe fibrosis as compared to AT_1 receptor knockout and wild-type mice in a model of interstitial fibrosis.[25] Similarly, it has been suggested that high levels of Ang-II following AT_1 receptor blockade stimulate non-engaged AT_2 receptors, and thereby influence extracellular matrix turnover, which may explain the decreased fibrosis observed in a rat model of ischemic myocardial fibrosis.[26]

Contrary to these observations, in our experiment the protective effect of long-term AT_1 receptor blockade even in the presence of additional AT_2 receptor blockade suggested that signalling via the AT_2 receptor is not of crucial importance for the delay of CAN. The differences between the models may be explained by variations in the expression of the two receptor subtypes with regard to tissue type, cell type, disease stage as well as the quality of receptor cross-talk.

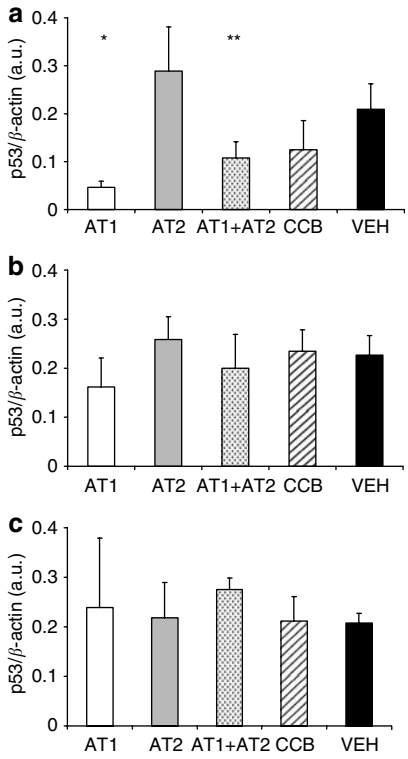

Figure 6 | Expression of p53. (**a**) Animals receiving *long-term* (day −7 before transplantation to week 24 after transplantation) AT_1 antagonist treatment (group AT1) had a significantly lower p53 expression as compared to animals of groups AT2 and VEH (*AT1 vs AT2: $P = 0.03$; vs CCB: $P > 0.05$; vs VEH: $P = 0.06$. **AT1 + AT2 vs AT2, CCB, VEH: $P > 0.05$). (**b**) Animals receiving *late* (week 12 to week 24 after transplantation) AT_1 and $AT_1 + AT_2$ antagonist treatment (groups AT1 and AT1 + AT2) had a lower expression as compared to animals of groups AT2, CCB, and VEH, respectively, but the differences were not significant ($P > 0.05$). (**c**) Animals receiving *early* treatment (day −7 before transplantation to day 5 after transplantation) had no significant differences between the groups ($P > 0.05$). □ = AT1: treatment with AT_1 receptor antagonist; ▩ = AT2: treatment with AT_2 receptor antagonist; ▨ = AT1 + AT2: treatment with AT_1 and AT_2 receptor antagonist; ▨ = CCB: treatment with CCB; and ■ = VEH: treatment with vehicle.

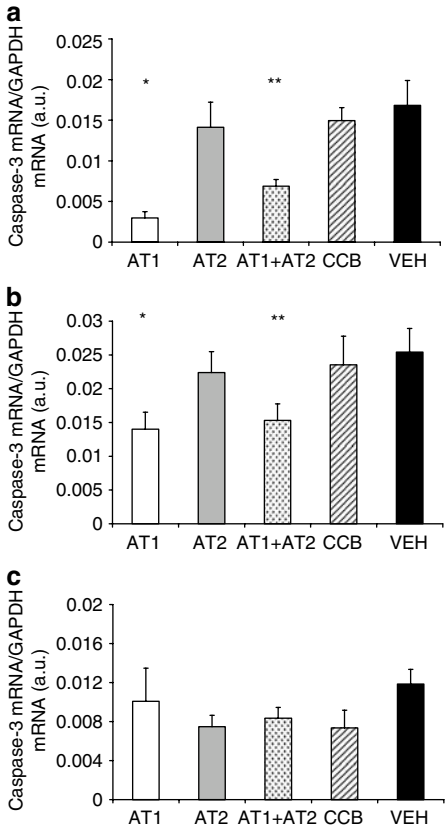

Figure 7 | Caspase-3 mRNA. (**a**) Animals receiving *long-term* (day −7 before transplantation to week 24 after transplantation) AT_1 antagonist treatment (group AT1) had significantly lower levels of caspase-3 mRNA as compared to groups AT2 and VEH (*AT1 vs AT2: $P = 0.018$; vs CCB: $P = 0.09$; vs VEH: $P = 0.018$). Animals of group AT1 + AT2 had significantly lower levels as compared to groups CCB and VEH (**AT1 + AT2 vs AT2: $P = 0.06$; vs CCB: $P = 0.03$; vs VEH: $P = 0.03$). (**b**) Animals receiving *late* (week 12 to week 24 after transplantation) AT_1 antagonist treatment (group AT1) had significantly lower caspase-3 mRNA levels as compared to animals of groups CCB and VEH (*AT1 vs AT2: $P = 0.09$; vs CCB: $P = 0.009$; vs VEH: $P = 0.006$. **AT1 + AT2 vs AT2: $P = 0.15$; vs CCB: $P = 0.09$; vs VEH: $P = 0.12$). (**c**) Animals receiving *early* treatment (day −7 before transplantation to day 5 after transplantation) had no significant differences ($P > 0.05$). □ = AT1: treatment with AT_1 receptor antagonist; ▩ = AT2: treatment with AT_2 receptor antagonist; ▨ = AT1 + AT2: treatment with AT_1 and AT_2 receptor antagonist; ▨ = CCB: treatment with CCB; and ■ = VEH: treatment with vehicle.

In accordance with our results are observations that AT_1 receptor-activating antibodies can promote vascular rejections that may eventually contribute to the progression of CAN.[27]

How did AT_1 receptor blockade delay the progression of CAN, particularly in the long-term-treated animals? Lowering blood pressure alone was not responsible for the favorable results of AT_1 receptor blockade as blood pressure did not significantly differ between the groups, which is in congruence with previous reports.[28,29]

Apoptosis might contribute to a decrease of graft function by a reduction of functioning nephrons. Interestingly, Ang-II can induce apoptosis in tubular epithelial cells through the AT_1[30] as well as the AT_2 receptor.[31] Thus, angiotensin receptor blockers could exert their protective effects also via an inhibition of apoptosis. Interestingly, Cao *et al.*[32] observed an inhibition of apoptosis, together with a protective effect, by the AT_2 receptor antagonist PD123319 in a model of subtotal nephrectomy. In another experiment, apoptosis of tubular cells was inhibited by an AT_2 receptor blocker in a short-term model of obstructive nephropathy.[22]

In our experiments, the lower proteinuria and grade of CAN was paralleled by a lower number of apoptotic tubular cells and a shift of the ratio of bcl-2/bax mRNA levels toward the antiapoptotic Bcl-2 in parallel to reduced caspase-3 mRNA levels (coding for caspase-3 that belongs to the effector molecules of apoptosis) in animals treated with the AT_1 receptor antagonist over the long term as compared to AT_2 receptor antagonist-treated animals and VEH-treated controls. Although mRNA levels should

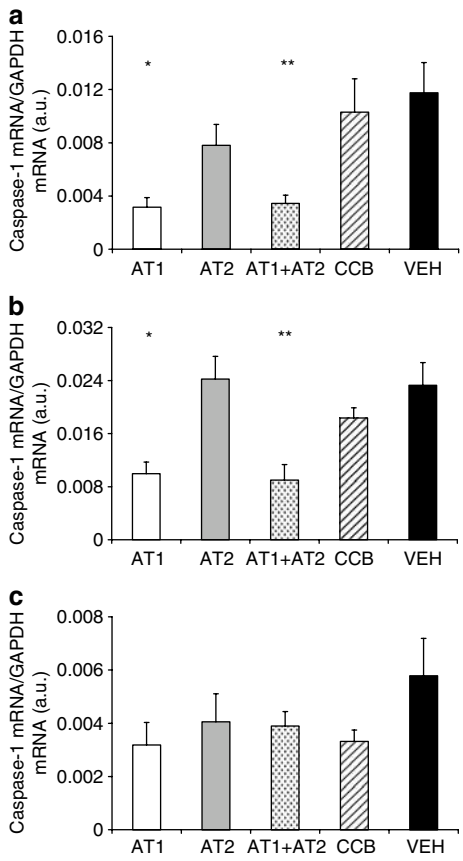

Figure 8 | Caspase-1 mRNA. (**a**) Animals receiving *long-term* (day −7 before transplantation to week 24 after transplantation) AT_1 antagonist treatment (group AT1) had significantly lower levels of caspase-1 mRNA as compared to groups AT2, CCB, and VEH (*AT1 vs AT2: $P = 0.027$; vs CCB: $P = 0.024$; vs VEH: $P = 0.012$. **AT1 + AT2 vs AT2: $P = 0.14$; vs CCB: $P = 0.09$; vs VEH: $P = 0.012$). (**b**) Animals receiving *late* (week 12 to week 24 after transplantation) AT_1 and $AT_1 + AT_2$ antagonist treatment (groups AT1 and AT1 + AT2) had significantly lower levels of caspase-1 mRNA as compared to groups AT2, and VEH (*AT1 vs AT2: $P = 0.03$; vs CCB: $P = 0.15$; vs VEH: $P = 0.03$. **AT1 + AT2 vs AT2: $P = 0.03$; vs CCB: $P = 0.09$; vs VEH: $P = 0.03$). (**c**) Animals receiving *early* treatment (day −7 before transplantation to day 5 after transplantation) had no significant differences, although levels were higher in animals of group VEH as compared to the other groups ($P > 0.05$). □ = AT1: treatment with AT_1 receptor antagonist; ▩ = AT2: treatment with AT_2 receptor antagonist; ▦ = AT1 + AT2: treatment with AT_1 and AT_2 receptor antagonist; ▨ = CCB: treatment with CCB; and ■ = VEH: treatment with vehicle.

be cautiously interpreted with respect to protein expression, the mRNA data in our model paralleled the number of apoptotic cells in the grafts. Thus, in our model, the AT_1 receptor seems to influence apoptosis more as compared to the AT_2 receptor.

However, whether apoptosis is involved in the pathogenesis of CAN is not clear so far. Data supporting a pathogenic role of apoptosis for renal tissue damage is derived from ischemia/reperfusion experiments, where inhibition of apoptosis protected kidneys from acute ischemia/reperfusion injury.[33] In kidney transplantation, observational studies have related apoptosis to CAN.[10] In models of unilateral ureteral obstruction, cell compartment-specific as well as time-dependent effects seem to influence apoptosis with different effects regarding interstitial volume or tubular atrophy for example.[34]

In our series, long-term AT_1 receptor blockade was associated with a reduced number of apoptotic cells and an amelioration of CAN, whereas the effects of late AT_1 receptor blockade on apoptosis and CAN were not significant. On the other hand, our group previously showed that late administration of the mammalian target of rapamycin inhibitor everolimus, known to induce apoptosis in immune cells and activated non-immune cells, also ameliorated CAN, which was associated with an increased number of apoptotic cells.[35] Conversely in a model of renal ischemia/reperfusion, early administration of the mammalian target of rapamycin inhibitor sirolimus also increased the number of apoptotic cells, but augmented renal damage.[36] It is difficult to compare the results of different studies as observation times, dosing of the drugs, and potential different effects of the drugs on apoptosis related to the type of the cell and its proliferation status might influence the overall effect. Further studies are needed to clarify what role apoptosis plays in progressive renal diseases and whether apoptosis could also be associated with varying effects at different periods during their progression.

In our study, the differences in the number of apoptotic cells were probably not related to cellular proliferation in the graft tissues, as no significant differences in the number of proliferating cells were present.

Interestingly, animals receiving long-term AT_1 receptor antagonist treatment had lower caspase-1 mRNA levels together with a reduced macrophage infiltration of the graft tissue as compared to controls. As caspase-1 has been associated with apoptosis[37] and inflammation,[38] a link between those processes may exist. Thus, hypothetically, the function of caspase-1 (apoptotic/inflammatory) could depend on the surrounding environment.

What could be the mechanisms responsible for a reduction of apoptosis by inhibition of the AT_1 receptor? One of the pathways by which Ang-II can induce apoptosis is an upregulation of p53 that in turn can increase the number of AT_1 receptor molecules, and, moreover, shifts the Bcl-2/Bax ratio toward the apoptosis-promoting Bax.[21,39]. This is supported by our findings in animals receiving long-term AT_1 receptor blockade, as these animals had a reduced expression of p53 as compared to AT_2 receptor antagonist-treated animals.

Apart from a direct effect on the reduction of apoptosis in the grafts, indirect effects such as a decrease in proteinuria may also have accounted for the beneficial effects of AT_1 receptor blockade, as proteinuria is known to induce apoptosis.[40] Thus, it cannot be excluded that the number of apoptotic cells has additionally been affected through a reduction of proteinuria as a result of AT_1 receptor antagonist treatment.

In summary, long-term AT_1 receptor blockade delayed CAN more effectively as late AT_1 receptor blockade, while AT_1 receptor signalling seems to be more important than signalling via the AT_2 receptor. Clinical trials are needed to analyze whether an early initiation of AT_1 receptor blockade could delay CAN in humans more effectively than delayed blockade. The protective effects of AT_1 receptor blockade may be related to a lower number of apoptotic tubular epithelial cells in the graft related to the lower expression of p53.

MATERIALS AND METHODS

Animals, surgery, and treatment

Kidneys of male Fisher rats (F344, $RT1^{lvl}$) (170–210 g) (Charles River, Sulzfeld, Germany) were transplanted into male Lewis rats (LEW, $RT1^{l}$) (170–210 g) (Charles River, Sulzfeld, Germany).[41] The left native recipient kidney was removed and the left donor kidney was orthotopically transplanted by end-to-end anastomosis of renal artery, vein, and ureter under inhalative anesthesia with isoflurane. All animals received cyclosporin A subcutaneously (1.5 mg/kg/day; Sigma-Aldrich, Taufkirchen, Germany) from day 0 to 10 after transplantation, when the remaining right kidney was removed. Animals received no other immunosuppressive drugs (e.g. steroids).

We assigned animals to five groups (Table 1): AT1 (AT_1 receptor antagonist candesartan, 5 mg/kg/day); AT2 (AT_2 receptor antagonist PD123319, 1.5 mg/kg/day); AT1 + AT2 (candesartan and PD123319); the control groups received either vehicle (VEH) or the calcium channel blocker (CCB) lacidipine (1 mg/kg/day) to control for the potential blood pressure lowering effect of candesartan.

The different treatments were applied for various periods: *long-term treatment* (day −7 to week 24 after transplantation; $n = 8$/group); *late treatment* (week 12–24 after transplantation; $n = 8$/group), and *early treatment* (day −7 to day 5 after transplantation; $n = 6$/group). The observation ended after 24 weeks. In the respective groups, administration of the substances was initiated 7 days before transplantation in donors and recipients to achieve the maximum pharmacodynamic effect at transplantation.

Functional measurements

We determined 24-h urine protein excretion every 4 weeks.[41] Serum creatinine levels were analyzed by an automatic laboratory analyzer (Synchron CX5, Beckman Coulter, Krefeld, Germany). Creatinine clearance was calculated according to the formula ($CrCl = U_{crea}$ (mg/dl)*diuresis (ml)/S_{crea} (mg/dl)*1440 (min)/weight (kg)). Mean arterial blood pressure was measured intra-arterially before the removal of the graft by a digital analyzer using a pressure transducer (Siemens, Munich, Germany). Measurement was performed under standard conditions at 24°C room temperature between 0900 and 1200 hours.

Morphological studies

Paraffin-embedded tissue sections were stained with hematoxylin and eosin to evaluate tubulointerstitial fibrosis, vasculopathy, and tubular atrophy. CAN was graded according to parameters adapted from the BANFF 97 classification:[2] 0 = no signs of CAN; grade 1 = mild CAN (mild fibrosis and tubular atrophy, 5–15% of section); grade 2 = moderate CAN (moderate fibrosis and tubular atrophy, 16–50% of section); and grade 3 = severe CAN (severe fibrosis and tubular atrophy, >50% of section). Grading also included vasculopathy with mild vasculopathy (intimal proliferation with luminal obstruction ⩽25%), moderate vasculopathy (luminal obstruction >25–50%), and severe vasculopathy (luminal obstruction >50% of section). Glomerulosclerosis was analyzed by counting sclerotic and normal glomeruli of the section and given as the percentage of sclerotic glomeruli.

Macrophage infiltration was evaluated with the alkaline phosphatase antialkaline phosphatase complex (Dako, Glostrup, Denmark) after incubation of cryostat sections (4 μm) with mouse anti-CD 68 (Serotec, Wiesbaden, Germany) and secondary rabbit anti-mouse IgG followed by development with fast red chromogene solution (Dako, Glostrup, Denmark). Cells with positive staining were counted and infiltration was graded: 0 = <5 cells/field of view; 1 = 6–25 cells/field of view; 2 = 26–50 cells/field of view; and 3 = >50 cells/field of view. At least 20 fields of view per section and per specimen were evaluated at ×400 magnification. Two independent observers evaluated the samples in a blinded manner.

The number of proliferating cells was analyzed after incubation of the sections with mouse anti-proliferating cell nuclear antigen antibody (Dako, Glostrup, Denmark) followed by incubation with a secondary horse anti-mouse antibody according to the Avidin Biotin Complex Method and development of the color signal by peroxidase (Vectastain elite Avidin Biotin Complex Kit; Vector Laboratories, Burlingame, CA, USA). Tubular epithelial cells with nuclear brown staining were counted per field of view at ×100 magnification.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assays were performed in frozen sections incubated with TUNEL solution[42] (Roche Diagnostics, Mannheim, Germany). After washing, sections were incubated with a rabbit anti-digoxygenine antibody (Roche Diagnostics, Mannheim, Germany). Binding was visualized using fast red chromogene solution. Tubular epithelial cells with positive staining (red nuclei) were counted per field of view. At least 20 fields of view per section and per specimen were evaluated at ×400 magnification. Two independent observers evaluated the samples in a blinded manner.

Ribonuclease protection assay

Total RNA was prepared as previously described for the analysis of mRNA levels of Bcl-2, Bax, caspase-1, and caspase-3.[41] A substantial part of renal tissue consists of cortical and medullar tubular cells (63 and 34% of relative renal tissue volume).[43] Thus, a gross part of the isolated mRNA is derived from tubular cells. Radioactive antisense riboprobes (^{32}P-UTP) were prepared by *in vitro* transcription (RiboQuant Multi-Probe Ribonuclease Protection Assay (RPA) System, Pharmingen, San Diego, CA, USA)[35] followed by hybridization of total RNA samples with their respective rat antisense riboprobes according to the manufacturer's protocol. Then, RNA and RNase digestion and separation on a 6% polyacrylamide gel were performed. Blots were scanned on a Fuji-BAS phosphor imager (Fuji, Düsseldorf, Germany), digitized, and density of bands was measured. The ratio between specific mRNA band densities and housekeeping gene mRNA band (glyceraldehyde-3-phosphate dehydrogenase) densities were calculated.

Expression of p53

Tissue samples were homogenized in lysis buffer and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Samples were transferred to Hybond-CExtra membranes (Amersham Pharmacia Biotech, Aylesbury, UK), blocked with 5% skimmed milk in Tris-buffered saline/0.5% Tween 20 overnight at 4°C.

Membranes were incubated with the following antibodies after washing in Tris-buffered saline/Tween: primary antibodies – monoclonal mouse anti human p53 (1:5000; Pharmingen, San Diego, CA, USA), polyclonal rabbit anti β-actin (1:10 000; Sigma, St Louis, MO, USA); secondary antibodies – goat anti-mouse immunoglobulin G-horseradish peroxidase conjugated (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-rabbit immunoglobulin G-POD conjugated (1:5000; Vector Laboratories, Burlingame, CA, USA). Antibodies were diluted in 5% skimmed dry milk in Tris-buffered saline/Tween buffer. Blots were washed and incubated with LumiLight working solution (Roche Diagnostics GmbH, Mannheim, Germany). Density of bands was analyzed and the ratios between p53- and β-actin bands were calculated.

Statistical analysis

Data are expressed as mean $\pm$ s.e.m. Data were tested using the χ^2 test or Mann–Whitney *U*-test. Bonferroni's method was applied to avoid inflation of type I error in multiple testing settings. A global *P*-value of <0.05 was considered significant. All tests were performed two-tailed. Data were analyzed using the SPSS statistical software package (v. 13.0, SPSS GmbH, Munich, Germany).

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